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**14. ABSTRACT** This project ultimately aims to identify the role of inhibin alpha (INHA) in advanced prostate cancer. The hypothesis to be tested is that INHA is tumor promoting and pro-metastatic in advanced prostate cancer. To date, we have made significant progress towards understanding the role INHA in advanced prostate disease. We have demonstrated that increased INHA expression in highly aggressive, metastatic and androgen-independent prostate cancer cell line, PC3, further promotes its tumor growth and metastatic ability. Increase in metastasis was further evident by increase lymph vessel density (LVD) and lymphatic invasion by the cancer cells. This was also accompanied by increase in VEGF-A and VEGF-C expression. Furthermore, we conducted a cross-sectional study to determine a link between INHA expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa. Elevated expression of INHA in primary PCa tissues showed a higher risk of PCa patients being positive for clinicopathological parameters outlined above. This study is the first to demonstrate a pro-tumorigenic and pro-metastatic role for INHA in the androgen-independent stage of metastatic prostate disease. Our results also suggest that INHA expression in the primary prostate tumor can be used as a predictive factor for prognosis of PCa. Our preliminary work on understanding the mechanism suggests the involvement of ERK/MAPKsignaling pathway through which INHA promotes tumor growth and metastasis. Whatever the outcomes of these experiments are, we are sure to contribute significantly to our understanding of the role of INHA in the process of prostate carcinogenesis.

**15. SUBJECT TERMS**
Inhibin alpha, prostate cancer, metastasis, lymph nodes, VEGF-C, extra-capsular spread, ERK/MAPK

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## INTRODUCTION

This project ultimately aims to identify the role of inhibin-α subunit (INHA) in prostate carcinogenesis. To date the role of INHA in reproductive cancers is equivocal. This project seeks to test the hypothesis that increased expression of INHA in advanced prostate cancer (PCa) promotes tumor growth and the spread of cancer cells to the lymph nodes. If this hypothesis is proven, then INHA will be implicated as being one of the factors inducing metastatic disease. In addition, we will provide the biological mechanisms affected by increased INHA expression in prostate carcinogenesis. Clinical specimens will also be used to determine INHA expression and lymph node status in PCa patients. To date, we have made significant progress towards demonstrating that increased INHA expression promotes tumor growth and metastasis in advanced PCa, as discussed below.

## BODY

Task 1: To investigate the tumor promoting and pro-metastatic role of INHA using *in vitro* and *in vivo* models (Months 1-6).

a. Immunohistochemistry for human mitochondria on tissues (primary prostate tumors and lymph nodes (LNs)) harvested from study already completed will show the presence of human cells in the primary and secondary tumors.

We have completed the aims of Task 1a during the first six months of the project. Specifically, this involved using immunohistochemistry to show presence of human cells in the harvested tissues thereby validating our preliminarily observations; *in vivo* data from INHA over-expressing cells showed increased tumor size following orthotopic injection and a 3.5 fold increase (75% versus 20%) in the incidence of metastasis from the primary tumor to surrounding LNs compared to controls. Monoclonal human mitochondria antibody was used to determine the presence of human cells in the tumors (primary prostate tumors and LNs). We also used monoclonal R1 antibody to determine INHA expression in tumors. For detailed description of the methodology see Appendix 1.

Positive immunostaining for human mitochondrial protein confirmed that the primary and secondary tumors originated from intra-prostatic injection of human cells. INHA immunostaining was used to confirm INHA expression in tumors (Fig 1A & B; *left*). INHA over-expression in PC3 cells had no effect on orthotopic tumor take but a significant increase in the primary prostate tumor size ($p = 0.005$) was observed (Fig 1A; *middle & right*). INHA over-expression in PC3 significantly increased the incidence of lymph node tumors ($p = 0.0341$) and lymph node tumor size ($p = 0.0047$) compared to the empty vector (EV)-transfected clones (Fig 1B; *middle & right*).

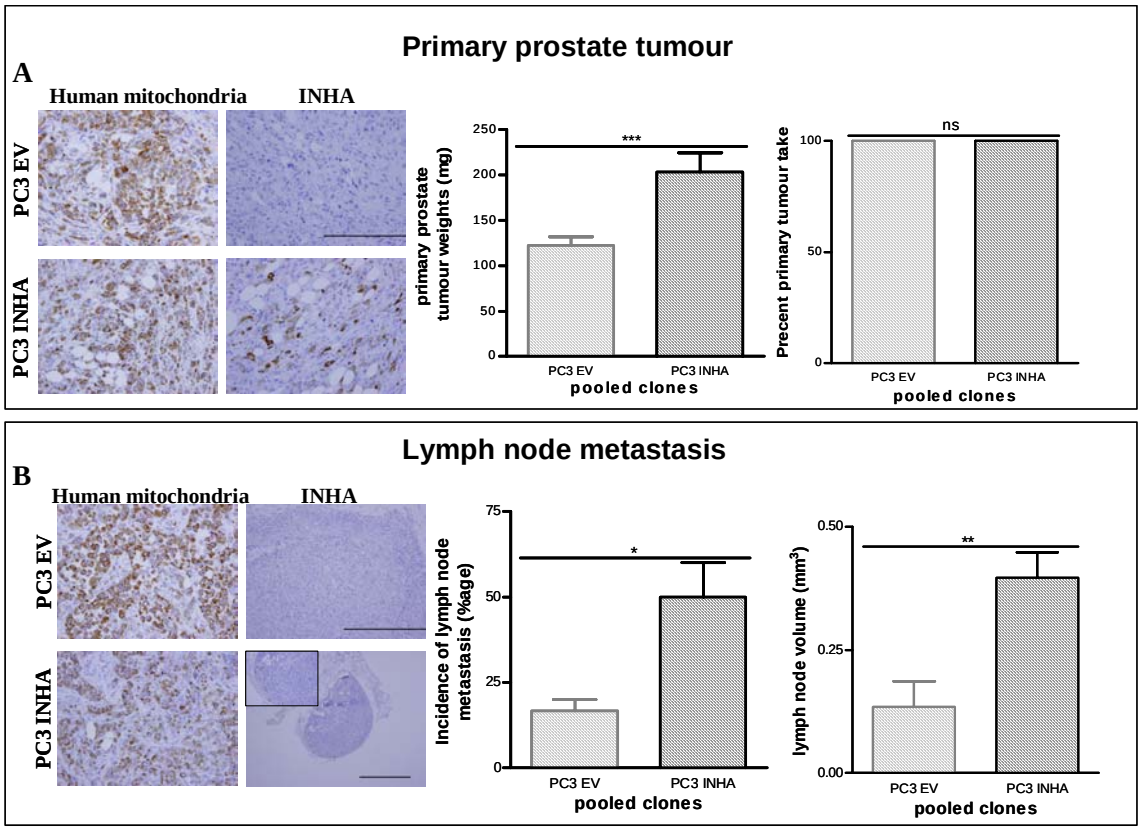


**Fig 1 Effect of INHA over-expression on primary prostate tumor growth and lymph node metastasis. A-B;** *left* Immunohistochemistry of primary prostate and lymph node tumors using human mitochondria and INHA staining confirmed the human origin of the cells in PC3 inoculated mice and INHA expression in the tumors. Bar 200 & 500μm. **A;** Primary prostate tumor weights *(middle)* and primary prostate tumor take *(right)*. **B;** Incidence of lymph node metastasis *(middle)* and lymph node volume *(right)*. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and no significant (ns) difference between the mean of the EV clones and the mean to the INHA-transfected clones. The bars represent: EV-transfected PC3 clones in grey, INHA-transfected PC3 clones in black. Data shown as mean ± SE of the mean.

b. Immunohistochemistry for human mitochondria and LYVE-1 on the primary prostate tumors will determine lymph vessel density (LVD) in the intratumoral, peritumoral and normal regions in the tissues.

We have completed the aims of Task 1a during the first six months of the project. Changes to LVD and lymphangiogenesis are often associated with metastatic spread of cancer cells to the LNs (1, 2). To understand the mechanisms and to provide proof of metastatic spread observed in the mice injected with INHA-positive cells we stained PC3 INHA and EV orthotopic tumors for LYVE-1, and human mitochondrial antibody to determine LVD and the degree of invasion of tumor cells into lymphatic vessels (lymphatic invasion) in the tissues (Fig. 2a). Stereological analysis of these tumors revealed a significant increase ($p = 0.0023$) in the LVD in the intratumoral regions with

no difference in LVD in peritumoral and surrounding non-malignant regions of INHA-positive tumors compared to the controls (Fig. 2b). Data also revealed significant increase in lymphatic invasion in the intratumoral (p = 0.0002), peritumoral (p = 0.0225) and non-malignant (p = 0.0077) regions of the tissue in INHA-positive tumors compared to the controls (Fig. 2c). For detailed description of the methodology see Appendix 1.

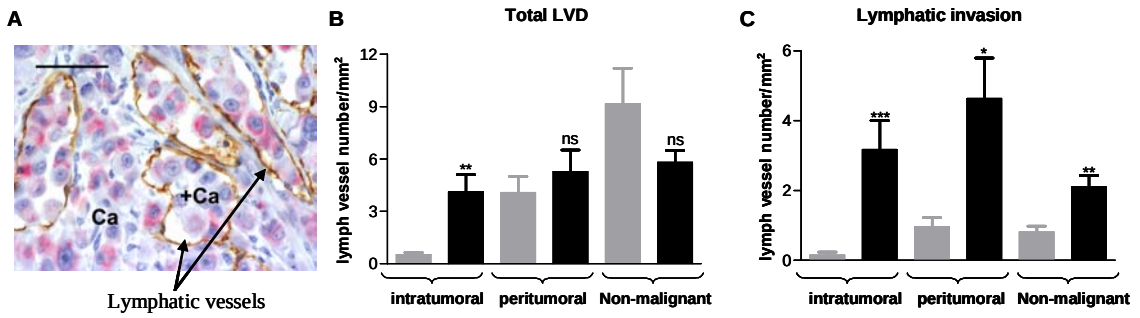


**Fig 2 Increase in lymphatic vessel density and lymphatic invasion in PC3 tumors.** *A*, Lymphatic vessels (LVs) were stained with LYVE-1 antibody (brown) and human prostate cells (Ca) with human mitochondria antibody (purple). Bar 50μm. The total number of LVs *(B)* and LVs with cancer cells in their lumen *(C)* (for example of such a vessel see "+Ca" in panel A) in the intratumoral, peritumoral and non-malignant (benign region adjacent to the tumor) regions of the primary prostate tumor were counted. * p 0.01 – 0.05, ** p 0.001 – 0.01, *** p < 0.001 and no significant (ns) difference between LVD in INHA over-expressing primary tumor compared to EV tumors. The bars represent: EV-transfected PC3 clones in grey, INHA-transfected clones in black. Data shown as mean ± standard error of the mean.

c. Collection of fresh prostate tissues from 3 prostate cancer patients, isolation of lymphatic endothelial cells (LECs) from and culturing them in the presence of PCa cells with and without INHA expression and/ or recombinant inhibin protein will determine the effect of the cancer cells and recombinant inhibin protein on LEC tube number and length.

Before this work begins, we were required to obtain Human Ethics approval to access human prostate tissues from patients undergoing radical prostatectomy surgery. Our laboratory already holds a human ethics approval at East Epworth Hospital, Boxhill, Melbourne, Australia to obtain fresh prostate tissues from patient undergoing surgery for another project "Role of tumor stroma in prostate carcinogenesis". The original application was amendment to include access of tissues for isolating human lymphatic endothelial cells. The primary approval was granted from Epworth Human Ethics committee (Approval Number: 34306 on 06 December 2006 [see appendix 2]. Secondary approval was granted from Monash University Standing Committee on Ethics in Research Involving Humans (Approval Number: 2004/145MC) on 13 June 2007 [see appendix 3], which was necessary since some staff involved in the project are employees of Monash University.

We have successfully completed practice experiments of isolating LECs from prostate tissue. There has been no other progress made for this aim at the present time.

Task 2: To determine the mechanism through which INHA may promote tumor growth and metastasis (Months 3 – 24).

a. ELISAs for VEGF-C and VEGF-D expression at the protein level will confirm changes in INHA over-expressing PC3 cells and empty vector (EV) transfected PC3 cells.

We have completed the aims of Task 2a during the first six months of the project. The observed increase in LVD in INHA-positive PC3 tumors suggested that the metastatic spread of the cancer cells from the primary tumor site to the LNs occurs through the process of lymphangiogenesis. Members of the vascular endothelial growth factor (VEGF) family, VEGF-C, VEGF-D and more recently VEGF-A, have been associated with lymphangiogenesis, mediating their effects through vascular endothelial growth factor receptors 2 and 3 (VEGF R2 and VEGF R3) (3-5). Our preliminary data showed that there was no change in VEGF-D mRNA levels in INHA over-expressing PC3 cells compared their EV controls, therefore it was decided not to analyze VEGF-D expression any further. However, we went on to determine the expression of VEGF-A and VEGF-C protein by ELISA, in INHA- and EV-transfected clones *in vitro*. VEGF-A mRNA levels were also determined. For detailed description of the methodology see Appendix 1.

VEGF-A ($p = 0.0002$) and VEGF-C ($p = < 0.0001$) mRNA levels were significantly increased in INHA over-expressing PC3 cells (Table 1). Secreted VEGF-C protein levels were significantly increased ($p = 0.0011$) in the INHA over-expressing PC3 clones compared to their EV clones, however there was no significant change in secreted VEGF-A levels (Table 1).

**Table 1 Effect of over-expressing INHA on VEGF-A and VEGF-C**

| | | |
|---|---|---|
| | **PC3** | |
| | **EV clones** | **INHA clones** |
| ***VEGF-A*** | | |
| normalised mRNA | 0.016 ± 0.002 | 0.0423 ± 0.006*** |
| protein (µg/µl) | | |
| cell lysate | 626.8 ± 25.04 | 500.9 ± 24.51* |
| conditioned media | 1794 ± 40.31 | 1712 ± 34.64 |
| | | |
| ***VEGF-C*** | | |
| normalised mRNA | 12.76 ± 1.59 | 34.96 ± 2.76*** |
| protein (µg/µl) | | |
| cell lysate | 319.6 ± 49.21 | 656.7 ± 46.21** |
| conditioned media | 3377 ± 566.0 | 6892 ± 531.6** |

* p = 0.01 - 0.05
** p = 0.01 - 0.001
*** p = < 0.001

b. Microarray technology, siRNA and small inhibiting molecules will comprehensively analyze and identify the pathway(s) inhibin affects in regulating its tumor promoting and pro-metastatic role.

We have successfully completed the first part of Task 2b i.e. we have used microarray technology to identify the pathway(s) inhibin affects in regulating its tumor promoting and pro-metastatic role. However, there was a modification to the initial proposal. At the time of the experiment, we decided to use Affymetrix GeneChip Human GENE 1.0ST array (Affymetrix) and TGFß BMP Signaling Pathway Oligo GEArray (Superarray) to get more cost effective, efficient and robust results instead of using several different signal pathway arrays.

Briefly, INHA and EV transfected PC3 cells were cultured using standard techniques. These cells were then prepared for analysis on the gene arrays according to the manufacturer's instructions. The TGFß BMP Signaling Pathway array experiments were performed at Monash. The Affymetrix GeneChip Human GENE 1.0ST array experiments were performed and analyzed in collaboration with Dr Robin Anderson and Dr Bedrich Eckhardt at the Cancer Biology Group, Peter MacCallum Cacner Centre, Melbourne, Australia. Table 2 shows only the most significant gene changes.

To specifically identify changes in the TGFβ pathway, the Affymetrix data was further analyzed using Ingenuity System. This identified ERK/MAPK pathway to be altered in our INHA-over-expressing cells compared to the EV cells. Analysis of the TGFβ BMP array also revealed changes in MAPK gene expression and few other genes that may link INHA expression to its tumor promoting role. We are currently in the process of validating the array results by real-time PCR and western blot. There has been no progress towards the rest of the aims at present.

c. RNA interference technology via a viral delivery system will be used to down-regulate INHA and VEGF-C expression in INHA over-expressing cells and orthotopic inoculation of these cells in mice will show and confirm that INHA stimulated VEGF-C is responsible for increased in LVD and increased incidence of LN metastasis. We project to use 100 male SCID mice for this study (10 mice per group).

Before studies on mice can begin, we were required to obtain Animal Ethics approval from Monash Medical Centre Animal Ethics Committee to use mice for this project. The approval was granted (Application number: MMCA 2006/45) on 20 March 2007 [see appendix 4]. We have successfully established viral expression and delivery system in our lab. However, there was significant delay towards commencing the main experiment. While we were able to produce virus, we had difficulty infecting our PCa cells and therefore creating knock-down clones. After several attempts it was decided to use traditional transfection protocols to produce the knock-down clones.

# Table 2 Difference in gene expression in INHA over-expressing PC3 cells compared to EV cells.

**Changes in gene expression using TGF BMP signaling pathway array**

| Gene symbol | Description | fold difference | p value |
|---|---|---|---|
| *Genes over-expressed* | | | |
| MAP3K7 | Mitogen-activated protein kinase kinase kinase 7 | 2.48 | 0.028 |
| TGIF1 | TGFB-induced factor homeobox 1 | 2.07 | 0.005 |
| MYC | V-myc myelocytomatosis viral oncogene homolog (avian) | 2.05 | 0.064 |
| TGFB2 | Transforming growth factor, beta 2 | 2.00 | 0.043 |
| MAP3K7IP1 | Mitogen-activated protein kinase kinase kinase 7 interacting protein 1 | 1.78 | 0.003 |
| INHBA | Inhibin, beta A (activin A, activin AB alpha polypeptide) | 1.58 | 0.419 |
| SMURF1 | SMAD specific E3 ubiquitin protein ligase 1 | 1.57 | 0.036 |
| SMURF2 | SMAD specific E3 ubiquitin protein ligase 2 | 1.54 | 0.106 |
| *Genes under-expressed* | | | |
| RUNX2 | Runt-related transcription factor 2 | 0.64 | 0.202 |
| LASS1 | LAG1 homolog, ceramide synthase 1 (S. cerevisiae) | 0.42 | 0.186 |
| GDF3 | Growth differentiation factor 3 | 0.40 | 0.158 |

**Changes in gene expression using Affymetrix array**

| Gene symbol | Description | fold difference | p value |
|---|---|---|---|
| BEX1 (includes EG:55859) | brain expressed, X-linked 1 | 9.35 | 32.78 |
| INHA | inhibin, alpha | 4.10 | 3.55 |
| DPP4 | dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2) | 4.90 | 2.48 |
| TSPAN12 | tetraspanin 12 | 1.53 | 2.19 |
| SNX16 | sorting nexin 16 | 2.19 | 2.03 |
| ASRGL1 | asparaginase like 1 | 2.04 | 2.02 |
| PLP2 | proteolipid protein 2 (colonic epithelium-enriched) | 1.56 | 1.89 |
| TRPS1 | trichorhinophalangeal syndrome I | 1.55 | 1.87 |
| PAPSS1 | 3'-phosphoadenosine 5'-phosphosulfate synthase 1 | 1.53 | 1.83 |
| PLCB4 | phospholipase C, beta 4 | 2.83 | 1.79 |
| SPATA6 | spermatogenesis associated 6 | 1.52 | 1.77 |
| TUBA1A | tubulin, alpha 1a | 3.99 | 1.75 |
| ERV3 (includes EG:2086) | endogenous retroviral sequence 3 (includes zinc finger protein H-plk/HPF9) | 1.67 | 1.74 |
| LOC100133941 | CD24 molecule | 6.66 | 1.70 |
| LOC158160 | hydroxysteroid (17-beta) dehydrogenase 7 pseudogene 2 | 1.59 | 1.67 |
| NRIP1 | nuclear receptor interacting protein 1 | 1.54 | 1.63 |
| MYEF2 | myelin expression factor 2 | 3.82 | 1.62 |
| CACNB3 | calcium channel, voltage-dependent, beta 3 subunit | 2.00 | 1.60 |
| ZNF816A | zinc finger protein 816A | 1.77 | 1.59 |
| SERPINE2 | serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 | 1.63 | 1.54 |
| GRAMD3 | GRAM domain containing 3 | 1.61 | 1.52 |
| SPECC1 | sperm antigen with calponin homology and coiled-coil domains 1 | 1.60 | 1.51 |
| STARD4 | StAR-related lipid transfer (START) domain containing 4 | 2.29 | 1.51 |
| LOC646853 | hypothetical LOC646853 | 1.72 | 1.50 |
| | | | |
| PLA2G4A | phospholipase A2, group IVA (cytosolic, calcium-dependent) | -3.45 | -1.54 |
| HOXB9 | homeobox B9 | -1.62 | -1.55 |
| LCN2 | lipocalin 2 | -2.19 | -1.57 |
| ADCY1 | adenylate cyclase 1 (brain) | -1.52 | -1.61 |
| WSB1 | WD repeat and SOCS box-containing 1 | -1.58 | -1.62 |
| ERO1L | ERO1-like (S. cerevisiae) | -2.33 | -1.63 |
| NMD3 | NMD3 homolog (S. cerevisiae) | -1.62 | -1.68 |
| ADM | adrenomedullin | -1.53 | -1.71 |
| HK2 | hexokinase 2 | -1.84 | -1.87 |
| RAB31 | RAB31, member RAS oncogene family | -2.19 | -2.07 |
| PXDN | peroxidasin homolog (Drosophila) | -1.78 | -2.26 |
| TFPI | tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) | -1.55 | -2.31 |

To date we have successfully created INHA knock-down clones and INHA expression expression levels were confirmed by radio-immunoassay Table 3. However, even after several attempts we have not managed to create VEGF-C knock-down clones. There has been no progress towards the rest of the aim at present.

**Table 3 INHA knock-down clones selected for further analysis**

| **PC3 cells** | **Inhibin (ng/ml)** |
|---|---|
| *INHA knock-down clones* | |
| P20/904/1 | <0.12 |
| P20/904/2 | <0.12 |
| P20/904/3 | <0.12 |
| P20/905/1 | <0.12 |
| *control clones* | |
| P20/NT/5 | 0.63 |
| P20/0.1/5 | 0.31 |
| P20/0.1/6 | 0.34 |
| *parental INHA-over-expressing cells* | |
| P20 | 0.30 |

d. Orthotopic inoculation of INHA and empty vector (EV) transfected PC3 cells followed regular injections of neutralizing antibodies will be used to block VEGF receptor signaling in the cells. This will show that by blocking VEGF receptor signaling we can reduce LVD and the incidence of LN metastasis. We project to use 60 male SCID mice for this study (10 mice per group).

We have completed the aims of Task 2d. There had been a delay towards this aim since we were unable to obtain the neutralizing antibodies (VEGF R2 and VEGF R3) to block VEGF receptor signaling in the cells as originally planned. To minimize this having an effect on this project and delaying the experiments further we formed a collaborative research with Schering AG, Corporate Research Oncology who has sent us PTK/ZK, a compound known to specifically block VEGF receptor signaling (6). PTK/ZK has successfully been used in both animal experiments (6) and human clinical trial for advanced colorectal cancer, acute myeloid leukemia and liver metastases (7, 8).

Before this work begins, we were required to obtain Animal Ethics approval from Monash Medical Centre Animal Ethics Committee to use mice for this project. The approval was grants (Application number: MMCA 2006/45) on 20 March 2007 [see appendix 4]. The use of this compound instead of the neutralizing antibodies also reduced the number of mice to be used for this aim. We used 40 male SCID mice for this study (10 mice per group).

*Method:* INHA- and EV-transfected cells were cultured using standard tissue culture techniques. These cells were injected into the prostate of 6 – 8 week old male SCID mice ($10^5$ cells/ injection) with the aim of forming tumors. Upon establishment of tumors, PTK/ZK was administrated orally per day for a period of 30-35 days. Each of 4 groups included 10 mice (Table 4).

| Table 4 Animal groups for AIM 2d |
|---|
| EV transfected PC3 cell line (P128) |
| INHA transfected PC3 cell line (P20) |
| INHA transfected PC3 cell line (P20) under PTK/ZK treatment |
| EV transfected PC3 cell line (P128) under PTK/ZK treatment |

The mice were monitored over a period of 8 weeks after which the primary tumors and regional LNs were collected. Using standard histopathological techniques collected tissues were sectioned and subjected to immunohistochemical (IHC) and stereological analysis and the incidence of LN metastasis were determined.

*Expected results:*
PC3 cell line produce VEGF family members, therefore blocking VEGF-R using PTK/ZK treatment should reduce tumor growth and LN metastasis. Our P128 model is an EV clone, therefore a representative of PC3 parental line. We expect mice with P128 tumors and treated with PTK/ZK to have small tumors and low incidence of LN metastasis compared to those treated with vehicle. As for P20, we expect similar outcome which may or may not be significant due to significant increase in VEGF-A and VEGF-C post INHA-transfection (Table 1). Furthermore, vehicle treatment P128 and P20 tumors should have similar results as show in Fig 1.

*Experimental results:*
There are several inconsistencies in the results from the above experiment. The tumor take, tumor weight and incidence of LN metastasis of P128 and P20 tumors (Fig 3) are not consistent to previous observed results (Fig 1).

*Tumor take:* We have previously shown that tumor take was 100% in both mice injected with P20 (INHA++) and those injected with P128 (INHA-- clone) (ref to Fig 1). The current results from this aim show that the tumor take is low in mice injected with P20 compared to P128 (Fig 3A).

*Tumor weight:* Furthermore, we have previously shown that P20 (INHA++) had significantly larger tumors than P128 (INHA-- clone) (ref to Fig 1), hence INHA over-expression in PC3 cells was tumor promoting. In the current experiment we have observed smaller tumors in mice injected with P20 compared to those injected with P128 (Fig 3B).

*LN metastasis:* Similarly we have previously shown that the incidence of LN metastasis to be higher in mice injected with P20 (INHA++) compared to those injected with P128 (INHA-- clone) (ref to Fig 1). The current results show that the incidence of LN metastasis is low in mice injected with P20 compared to P128 (Fig 3C).

Despite the inconsistencies, PTK/ZK treatment of mice with P128 tumors was successful. These mice showed reduction in tumor take, the tumors were smaller in size and there was also a reduction in the incidence of LN metastasis compared vehicle treated mice.

From this part of the study we can conclude that PTK/ZK has the potential to be used in the treatment of PCa patients to reduce the spread of cancer cells from the primary prostate tumor site to the LNs.

As mentioned above P20 tumors did not respond as expected. There may be two possible explanations for this:

1. changes to the tumorigenic properties of P20 while in culture prevented it from being more aggressive than P128
2. PTK/ZK treatment failed to block tumor growth and metastasis of P20 tumors because the previously observed outcomes (not reproducible here) are independent of VEGF family driven metastasis.

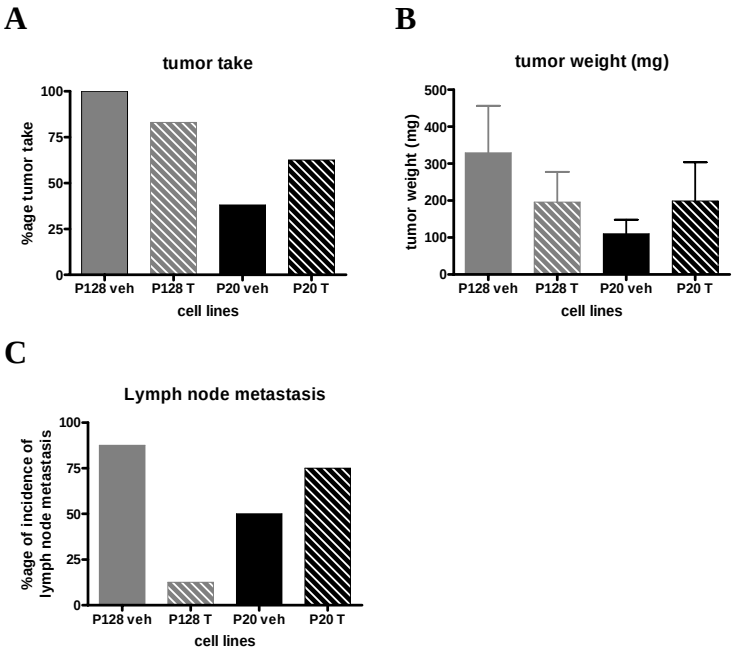


**Fig 3 Effect of blocking VEGF receptor signaling in PC3 cell lines.** The bars represent: EV-transfected PC3 clones in grey, INHA-transfected clones in black. Data shown as mean ± standard error of the mean.

Task 3: To determine the utility of INHA for the diagnosis of patients with highly aggressive and/ or metastatic PCa (Months 3-8)

a. Collection of archival human prostate tissue of at least 50 men who under radical prostatectomy. Tissues will be collected from patient who had organ-confined disease and those with lymph node metastasis.

We have completed the aims of Task 3a during the first six months of the project. Before this work begins, we were required to obtain Human Ethics approval to access archival human prostate tissues from patients who under went radical prostatectomy surgery. The

approval was granted from Monash University Standing Committee on Ethics in Research Involving Humans (Approval Number: CF07/0854 – 2007/0223HT) on 16 May 2007 [see appendix 5]. We collected archival tissues from patients who had organ-confined disease and those with lymph node metastasis.

b. Immunohistochemistry for INHA, VEGF-C and D2-40 will show the utility of INHA as a diagnostic or prognostic marker for PCa patients.

We have completed the aims of Task 3b during the first six months of the project. However, in the last 2-3 months we have re-evaluated the data for further analysis (see below). To reduce wastage of precious human prostate tissues it was decided to use a cohort of patient tissues that have already being evaluated for clinicopathological characteristics, VEGF-C expression and LVD (using D2-40) (1, 9) by our collaborating investigator; Elizabeth Williams. A number of independent studies have shown increases in INHA expression to be associated with PCa progression (10, 11). To determine if INHA expression can be correlated to lymph node status we used tissues from a cohort of PCa patients who had organ-confined disease and those who had lymph node metastasis. We obtained 20 radical prostatectomy specimens were from patients with organ-confined disease, while the remaining 16 specimens were from patients with LN metastases. The PO#12 antibody was used to determine the expression pattern of INHA in the prostate tissues. For detailed description of methodology see Appendix 1. An example of the INHA staining and scoring is provided in Fig 4.

Initial analysis of the immunostaining revealed significant increase in INHA staining in normal epithelial and intraepithelial neoplasia (PIN) regions with no change in the staining in the cancer regions of the prostate tumor tissues in patients with LN metastasis compared to patients with organ-confined disease (Fig 5A). However, there was no significant change in overall INHA expression in patients with LN metastasis compared to patients with organ-confined disease (Fig 5B).

We have recently re-evaluated the immunostaining and conducted a cross-sectional study to determine a link between INHA expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa (12-16). For detailed description of methodology and statistical analysis see Appendix 1. The immunostaining revealed differential expression of INHA in benign epithelial, G3/G4 cancer regions as well as in the stroma of primary PCa tissues from patients with organ-confined disease and those with metastasis to the lymph nodes (Fig 6). Association between clinicopathological prognostic factors and INHA expression are shown in Table 6. Elevated expression of INHA in the benign regions of the primary PCa tissues showed a higher relative risk in PCa patients been positive for extracapsular spread ($p = 0.01$). Similarly, elevated expression of INHA in the stroma of the primary PCa tissues showed a higher risk of PCa patients been positive for extracapsular spread ($p = 0.0011$), positive for surgical margins ($p = 0.0006$), positive for VEGF-R3 expression ($p = 0.00067$) and positive for lymph node metastasis ($p < 0.0001$). Further analysis showed that there was a

significant increase in INHA staining in benign ($p = 0.018$) and stromal ($p < 0.0001$) regions but not in G3/G4 cancer regions in tissues from patients with lymph node metastasis compared to patients with organ-confined disease (data not shown).

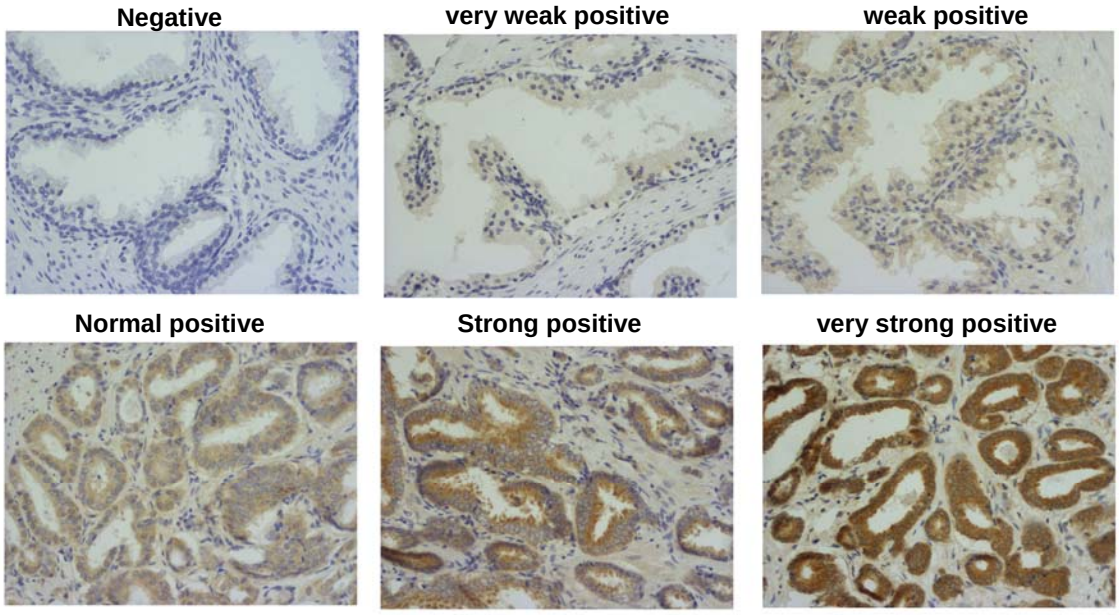


**Fig 4 Example of immunohistochemistry staining intensity used to evaluate the intensity of INHA staining in the prostate tissues.** Each immunostained tissue section was assessed and staining intensity in the different regions and grades of tumor was scored as following: -: negative (0); +/-: very weak positive staining (0.5); +: weak positive staining (1); ++: normal positive staining (2); +++: strong positive staining (3); ++++: very strong positive staining (4). Intensity of INHA staining in normal epithelial and intraepithelial neoplasia (PIN) and cancer regions (Gleason grade G1-G5) were analyzed to determine the pattern of INHA expression in the prostate tissues from patients with organ-confined disease and those with LN metastasis.

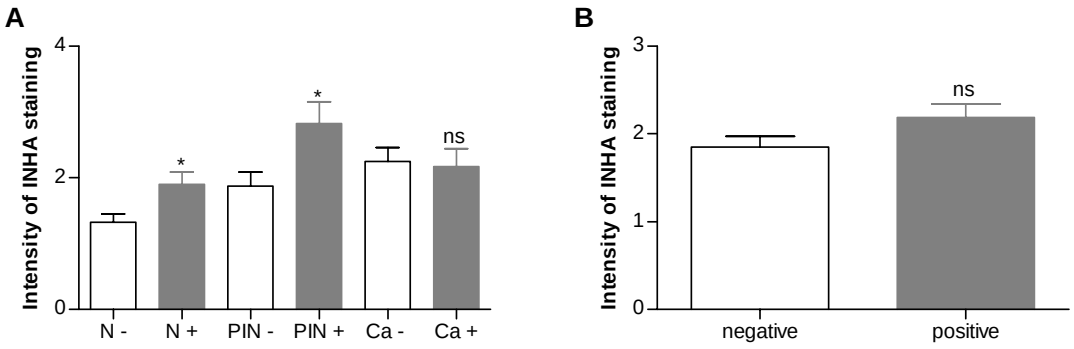


**Fig 5 INHA staining in PCa patients with organ confined and metastatic disease.** *A*, INHA immunostaining intensity in normal epithelial (N), PIN and cancer regions (Ca) was compared in patients with organ-confined prostate cancer (-) and those with

metastasis to the lymph nodes (+). * p 0.01 – 0.05 and no significant (ns) difference between the respective regions in organ confined and metastatic disease. *B.* Overall, there was no significant difference in INHA intensity in tissues from patients with organ-confined prostate cancer (negative) and those with metastasis to the LN (positive).

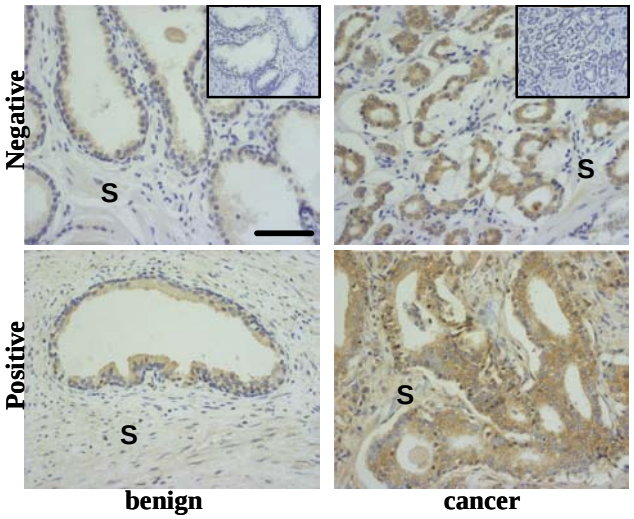


**Fig 6** INHA expression in clinical specimens and its association to prostate disease. Immunohistochemical staining of INHA in primary prostate tumors from PCa patients with organ confined (negative) and metastatic disease (positive). INHA immunostaining intensity in benign epithelial, cancer region (G3/G4) and stromal regions (S) are shown. Insert shows IgG control. Bar 200μM

**Table 6 Relationships between the expression of INHA and clinicopathological parameters in prostate adenocarcinoma (n = 37)**

| Parameters | No. of specimens | benign regions | | | Cancer regions (G3/G4) | | | stromal regions | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | mean intensity | Relative risk (95% CI) | p value | mean intensity | Relative risk (95% CI) | p value | mean intensity | Relative risk (95% CI) | p value |
| **combined Gleason grade** | | | | | | | | | | |
| 6 | 16 | 0.7 | n/a | | 1.88 | n/a | | 0.30 | n/a | |
| 7 | 14 | 1.51 | n/a | | 2.21 | n/a | | 1.21 | n/a | |
| ≥8 | 7 | 1.29 | n/a | | 1.99 | n/a | | 2.00 | n/a | |
| **extracapsular spread** | | | | | | | | | | |
| Positive | 24 | 1.43 | | | 2.13 | | | 1.41 | | |
| Negative | 13 | 0.65 (reference) | 2.07 (1.04 - 4.13) | 0.01^ | 1.87 (reference) | 1.27 (0.76 - 2.11) | ns^ | 0.08 (reference) | 2.55 (1.39 - 4.65) | 0.0011^ |
| **surgical margins** | | | | | | | | | | |
| Positive | 16 | 1.15 | | | 1.84 | | | 1.47 | | |
| Negative | 21 | 1.12 (reference) | 1.15 (0.68 - 3.08) | ns^ | 2.18 (reference) | 0.71 (0.32 - 1.53) | ns^ | 0.50 (reference) | 4.75 (1.62 - 13.93) | 0.0006^ |
| **VEGF-R3+ vessels** | | | | | | | | | | |
| Positive | 18 | 1.29 | | | 2.07 | | | 1.53 | | |
| Negative | 19 | 0.97 (reference) | 1.27 (0.62 - 2.60) | ns^ | 2.00 (reference) | 1.20 (0.0 - 2.39) | ns^ | 0.33 (reference) | 2.85 (1.28 - 6.37) | 0.0067^ |
| **Lymph node metastasis** | | | | | | | | | | |
| Positive | 16 | 1.41 | | | 2.06 | | | 1.93 | | |
| Negative | 21 | 1.01 (reference) | 1.62 (0.67 - 3.97) | ns^ | 2.00 (reference) | 0.84 (0.48 - 1.46) | ns^ | 0.15 (reference) | 13.22 (1.94 - 90.00) | p < 0.0001^ |

Abbreviations: VEGFR, vascular endothelial growth factor receptor; n/a, not applicable; ns, not significant; CI, confidence interval
^ Fisher's exact test

## RSEARCH ACCOMPLISHMENTS

- Demonstrated that over-expression of INHA enhanced metastatic ability of tumor cells *in vivo*.
- Gained Human Ethics approval for collection of fresh and archival human prostate tissues.
- Determined VEGF-A and VEGF-C expression at the protein level in INHA over-expression PC3 cells and the controls.
- Identified a possible signaling pathway inhibin affects in regulating tumor growth and metastasis.
- Created INHA knock-down clones for validation experiments.
- Obtained PTK/ZK, a compound which blocks VEGF family member signaling by blocking their receptor activity.
- Demonstrated the PTK/ZK treatment of mice with tumors can reduce tumor take, tumor size and incidence of metastasis to the LNs.
- Determined INHA expression pattern in PCa patients with organ-confined and metastatic disease.
- Demonstrated that increase in INHA staining in tissues from PCa patients is associated with a higher risk of surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa.

## REPORTABLE OUTCOMES

| | *Reportable outcomes that have resulted from this research:* |
|---|---|
| Manuscripts | Submitted to British Journal of Cancer. Due to the copyright issues only the title page, abstract and methodology used in the aims of this project is provided at this stage [see appendix 1] |
| Abstracts and presentations | Preetika Balanathan, Elizabeth D Williams, Hong Wang, Marc G Achen, Steven A Stacker, Gail Risbridger (2008) **Shift in the tumor suppressive activity of inhibin-α subunit during the transition from androgen-dependent to androgen-independent prostate cancer** TGFβ family in Homeostasis and Disease – Keystone Symposia, Santa Fe New Mexico, USA (poster presentation). [see appendix 6]<br><br>Preetika Balanathan, Elizabeth D Williams, Hong Wang, Marc G Achen, Steven A Stacker, Gail Risbridger (2008) **Tumor suppressive activity of inhibin-α subunit is altered during the transition from androgen-dependent to androgen-independent prostate cancer** Lorne Cancer, Victoria, Australia (poster presentation). [see |

| | |
|---|---|
| | appendix 7]<br><br>Preetika Balanathan (2008) **New action of inhibin-α in advanced prostate cancer** GlaxoSmithKline Post Graduate Support Grant award presentation day, Melbourne, Victoria. |
| Patents and licenses applied for and/or issued | Nil |
| Degrees obtained that are supported by this award | Nil |
| Development of cell lines, tissues or serum repositories | Nil |
| Informatics such as databases and animal models, etc | Nil |
| Funding applied for based on work supported by this award | GlaxoSmithKline (GSK) Post Graduate Support Grant for 2008 [see appendix 8]<br><br>ANZ Philanthropy Trust Fund Grant 2008 [see appendix 9]<br><br>Keystone Symposia – Travel scholarship [see appendix 10] |
| Employment or research opportunities applied for and/or received based on experience/training supported by this award | Nil |

## CONCLUSIONS

In summary, we have made significant progress towards understanding the role of INHA in advanced PCa. We have demonstrated increased tumor size and increased metastasis to the LNs by INHA over-expressing PC3 cells compared to the controls. The increase in metastasis was further evident by increase in total LVD and lymphatic invasion which was accompanied by increase in VEGF-C expression.

Using clinical specimens we have been able to determine that there is evidence of increased INHA expression in benign epithelial and stromal regions in tissues from PCa patients. This increase in INHA expression was significantly associated with higher risk of surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa.

Our initial work on understanding the mechanism suggests a role of ERK/MAPK signaling pathway through which INHA promotes tumor growth and metastasis. We will soon begin our work on confirming the significance of ERK/MAPK signaling pathway in

the pro-metastasis role of INHA. Whatever the outcomes of these experiments are, we are sure to contribute significantly to our understanding of the role of INHA in the process of prostate carcinogenesis.

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# APPENDIX 1 - MANUSCRIPT

## Elevated level of inhibin-α subunit is pro-tumourigenic and pro-metastatic and associated with extracapsular spread in advanced prostate cancer

Preetika Balanathan[1], Elizabeth D Williams[2], Hong Wang[1], John S Pedersen[3], Lisa G Horvath[4, 5], Marc G Achen[6], Steven A Stacker[6], Gail P Risbridger[1]

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*Short title:* Role of INHA in advanced PCa

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**Abstract**

The biological role of inhibin-α subunit (INHA) in prostate cancer (PCa) is currently unclear. A recent study associated elevated levels of INHA in PCa patients with a higher risk of recurrence. This promoted us to use clinical specimens and functional studies to investigate the pro-tumourigenic and pro-metastatic role of INHA. We conducted a cross-sectional study to determine a link between INHA expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa. In addition, using two human PCa cell lines (LNCaP and PC3) representing different stages of metastasis, this study investigated the biological role of elevated levels of INHA in advanced cancer. Elevated expression of INHA in primary PCa tissues showed a higher risk of PCa patients being positive for clinicopathological parameters outlined above. Over-expressing INHA in LNCaP and PC3 cells demonstrated two different and cell-type specific responses. INHA-positive LNCaP demonstrated reduced tumour growth while INHA-positive PC3 cells demonstrated increased tumour growth and metastasis via the process of lymphangiogenesis. This study is the first to demonstrate a pro-tumourigenic and pro-metastatic role for INHA in the androgen-independent stage of metastatic prostate disease. Our results also suggest that INHA expression in the primary prostate tumour can be used as a predictive factor for prognosis of PCa.

*Key Words:* inhibin-α subunit, prostate cancer, metastasis, androgen-independent

## Materials and Methods

### *Analysis of clinical material*

### *Relationship between INHA expression and clinicopathological parameters in primary prostate adenocarcinomas*

This study was conducted in accordance with Australian National Health and Medical Research Council (NH&MRC) Guidelines. Archival formalin-fixed paraffin-embedded tissue blocks were retrieved from 37 patients with prostate carcinoma who underwent radical prostatectomy. The clinicopathological characteristics, vascular endothelial growth factor-C (VEGF-C) expression, LVD and lymph node status of this cohort have been described previously (Zeng *et al*, 2004; Zeng *et al*, 2005). We conducted a cross-sectional study to determine whether INHA expression was associated with clinicopathological parameters (Gleason score, surgical margins, extracapsular spread, VEGF-R3 expression and lymph nodes status) and/or linked to well established prognostic factors in prostatic adenocarcinoma. The PO#12 antibody (kindly provided by Dr Nigel Groome) was used to determine the expression pattern of INHA in primary prostate tissues as previously described (Risbridger *et al*, 2004b). Each immunostained tissue section was assessed and staining intensity in benign epithelial,, cancer (Gleason grade G3/G4) and stromal regions was scored from 0 - 4 with 0 representing negative staining and 4 representing very strong positive staining. The relative risk of PCa patients being positive for the respective parameters was determined.

***Stable Transfection of LNCaP and PC3 cell lines***

LNCaP and PC3 were obtained from American Type Culture Collection (Rockville, MD) and routinely cultured as described previously (Balanathan *et al*, 2004). Expression vector pcDNA3.1 (empty vector (EV)) and human *INHA* cDNA subcloned into pcDNA3.1 (pcDNA3.1 (INHA)) were purchased from Invitrogen (Mount Waverley, Victoria, Australia) and prepared for transfection according to the manufacturer's instructions. LNCaP and PC3 cells were transfected using Lipofectamine plus (Invitrogen) and Superfect (Qiagen, Doncaster, Victoria, Australia), respectively according to the manufacturer's instructions. Individual colonies surviving after 2-3 weeks selection were picked and propagated for analysis.

***Confirmation of mRNA expression in INHA-transfected LNCaP and PC3 cell lines***

Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription (RT) was performed as previously described (Balanathan *et al*, 2004). $\beta_2$-microglobulin (*$\beta_2$mg*) was used as a housekeeping gene for block PCR. Primer sequences were: INHA Forward–CCTGTTCTTGGATGCCTTG, Reverse–AGCTGGGCTGAAGTCACCT and $\beta_2$mg Forward–CCGTGTGAACCATGTGA CTT, Reverse–CAAACATGGAGACAGCACTC. Absolute quantitative real time analysis was used to assess the levels of TGFβ receptor III (TGFβRIII) mRNA expression in the clones. The analysis was performed on a Lightcycler real-time PCR machine (Roche Diagnostic, Mannheim, Germany) using Lightcycler Fast Start DNA Master SYBR Green 1 (Roche Diagnostic) according to the manufactures instructions. All experiments were carried out twice and duplicate readings

were taken for each replicate. The quantity of mRNA was determined using a standard curve and all values were normalized using the house keeping gene, Hypoxanthine Ribosyl transferase (HPRT). Primer sequences were: TGFβRIII Forward-TTCCCTGTTCACCCGACCTGAAAT, Reverse-CGTCAGGAGGCACACA
TTA and HPRT Forward-TGTAATGACCAGTCAACAGGG, Reverse-TGGCTTATATC CAACACTTCG.

***Confirmation of protein expression by ELISA***

Cell lysates and conditioned media (conditioned for 24hrs) were prepared from EV- and INHA-transfected clones. Total protein (1 μg/μl) was used for further analysis. Inhibin A and B and activin A concentrations were measured in triplicates using specific ELISA according to the manufacturer's instructions (Diagnostic Systems Laboratories, Webster, TX). VEGF-A and VEGF-C ELISAs were measured in duplicate using specific ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). Two biological replicates were examined.

***Direct cell counting – proliferation assay***

LNCaP and PC3 cells were seeded at a density of $1x10^5$ cells/well and $5x10^3$ cells/well, respectively, in 24-well plates and incubated at 37°C. Triplicate wells were harvested by trypsinization on days 1, 2, 3, 4, and 5, and numbers of cells/well were counted using haemocytometer. Each experiment was repeated twice. The results obtained from individual clones (EV and INHA) were pooled for each treatment.

***Scratch wound assay – motility assay***

Cells were plated in triplicate in 6 or 12 well plates and grown until approximately 70-80% confluence. The cell monolayer was then wounded and analyzed over time as previously described (Sharp, 2004). Each experiment was repeated twice. The results obtained from individual clones (EV and INHA) were pooled and for each treatment.

***Intra-prostatic inoculation of LNCaP and PC3 cells***

The experiments were in accordance with NH&MRC of Australia Guidelines. LNCaP ($2x10^6$) or PC3 ($5x10^5$) clones were injected orthotopically into the ventral lobe of the prostate gland (10 animals/clone) of male SCID mice as previously described (Zeng *et al*, 2006). After 7-9 weeks, mice were killed and primary prostate tumours removed and weighed. In addition, regional lymph nodes were removed for analysis. Monoclonal human mitochondria antibody (1:100; Chemicon, Temecula, CA) was used to determine the presence of human cells in the tumours as previously described (McCulloch *et al*, 2005). The monoclonal R1 antibody (7.5 μg/ml), kindly provided by Dr Nigel Groome, was used to determine INHA expression in tumours as previously described (Balanathan *et al*, 2004).

Lymph node volumes were determined using stereological analysis as previously described (McPherson *et al*, 2001). The lymph nodes were serially sectioned at 5μm thickness and using a random sampling scheme, every $20^{th}$ section was chosen for analysis. Briefly, the computer program newCAST component (version 2.14; Visiopharm, Hørsholm, Denmark) was used to generate a point grid, and volumes of the lymph nodes were determined. Each section was examined under 20X magnification and

tissue sections were mapped to define tissue boundaries and were sampled at predetermined intervals along *x*- and *y-axes* using a single grid-counting frame. The volume was then determined using the equation = no of points for each tissue * area per point * distance; in this case the distance was defined by thickness of the sections (5μm) plus (5 μm * 20 for every $20^{th}$ section).

***Lymphatic vessel density (LVD) in the intra-prostatic tumours***

Lymphatic vessels were identified using lymphatic vascular endothelial hyaluronan receptor (LYVE-1), a marker of lymphatic endothelium (Banerji *et al,* 1999). Invasion of tumour cells into lymphatics was monitored by the presence of human mitochondrial protein stained cancer cells in lymph vessels. Double immunostaining for LYVE-1 and mitochondria was performed on a DAKO Autostainer (DAKO, Denmark). The sections were incubated with LYVE-1 antibody (Fitzgerald, MA) diluted at 0.5 μg/ml for 2 hours. LYVE-1 was detected by incubation with Envision polymer-anti-rabbit-HRP (DAKO) for 15min and visualised with diaminobenzidine (DAKO). Sections were then incubated with Double Staining Enhancer (Zymed, San Francisco, CA) for 15min and exposed to mitochondrial antibody (Chemicon) diluted at 1/200 for 2 hours. Secondary antibody, biotinylated rabbit-anti-mouse IgG1 (Zymed) was applied and the immunoreactivity was detected by ExtrAvidin-Alkaline phosphatase (Sigma, USA) and visualized by reaction with Vector-red (Vector Laboratories, CA). The sections were counterstained with Hematoxylin (DAKO) and immunolocalization was examined using an Olympus BX-60 microscope.

Lymphatic vessels were counted using stereological methods as previously described (Balanathan *et al*, 2004). Lymphatic vessels were counted within tissue sections (of randomly selected INHA-positive prostate tumours, n=15 and EV tumours, n=11; using n=2 randomly selected sections per tumour) to assess the LVD within the tumour (intratumoural) region, the region in contact with both the tumour and the stroma (peritumoural) and the region away from tumour. LVD was expressed as the number of lymph vessels per millimetre squared.

***Statistical Analyses***

All statistical analyses were performed results were analyzed by ANOVA or t-tests. The relationships between INHA expression and clinicopathological parameters were evaluated by Fisher's exact test. The mean staining intensity of patients positive for each of the respective clinicopathological parameter was compared to the mean staining intensity (reference) of those patients who were negative. The relative risks and 95% confidence intervals (CI) were estimated.

# APPENDIX 2: EPWORTH HREC APPROVAL

Epworth Hospital
ABN 97 420 694 950
*Epworth Foundation*
89 Bridge Road Richmond Vic 3121 Australia T: 61 3 9426 6666 F: 61 3 9427 0353 www.epworth.org.au
Epworth

27 December 2006

Prof. Gail Risbridger
**c/o Courtney Bamford**
Centre for Urological Research
Monash Institute of Medical Research
27 Wright Street,
CLAYTON VIC 3168

Dear Prof. Risbridger,

**Re: ROLE OF TUMOUR STOMA IN PROSTATE CARCINOGENISIS**
**Epworth Study No 34306**

Thank-you for your requested amendments and correspondence dated the 15th of November 2006.

The Epworth Healthcare HREC accepted these amendments at their meeting on the 6th of December 2006.

Thank-you for keeping the committee updated on the progress of your study and we look forward to receiving your annual report in due course.

Yours sincerely,

Louise Grey
HREC Coordinator
Epworth Hospital
89 Bridge Rd.
RICHMOND VIC 3121

# APPENDIX 3: SCERH APPROVAL



From scerh <scerh@adm.monash.edu.au>

Sent Wednesday, June 13, 2007 12:59 pm

To Renea Taylor <Renea.Taylor@med.monash.edu.au> , Gail Risbridger <Gail.Risbridger@med.monash.edu.au> , Preetika Balanathan <Preetika.Balanathan@med.monash.edu.au>

Cc

Bcc

Subject Monash Human Ethics - 2004/145MC: Role of tumor stroma in prostate carcinogenesis - Request for Amendment

PLEASE NOTE: To ensure speedy turnaround time, this correspondence is now being sent by email only. If you would prefer a hard copy on letterhead, please contact the Human Ethics Office (9905 2076 or scerh@adm.monash.edu.au) and a hard copy will be posted to you.

We would be grateful if first-named investigators could ensure that their co-investigators are aware of the content of the correspondence.

Dr Renea Taylor
Centre for Urological Research
Faculty of Medicine, Nursing and Health Sciences
Monash Medical Centre Campus

13 June 2007

2004/145MC: Role of tumor stroma in prostate carcinogenesis

Dear Researchers,

Thank you for submitting further information as requested by the Standing Committee on Ethics in Research Involving Humans (SCERH) with respect to the Request for Amendment to the above named project.

This is to advise that the requested amendments dated 2 April 2007, received in our office on 5 April 2007, have been approved and the project can proceed according to your approval given on 22 April 2004.

Monash SCERH therefore approves your submission approved by the Epworth Human Ethics Committee, for Epworth study 34306, on 6 December 2006.

Thank you for keeping the Committee informed.

Mrs Lyn Johannessen
Acting Human Ethics Officer (on behalf of SCERH)

Cc: Prof Gail Risbridger, Dr Preetika Balanathan

# APPENDIX 4: MMCA APPROVAL

MONASH INSTITUTE OF MEDICAL RESEARCH
AN INITIATIVE OF MONASH UNIVERSITY AND SOUTHERN HEALTH

Director:
Professor Bryan Williams

Executive Director:
Professor Adrian Walker

Centre Directors:
Professor Paul Hertzog
Professor Stephen Holdsworth
Dr Michael Holland
Professor Martin Pera
Professor Gail Risbridger
Associate Professor Peter Rogers

Patrons:
Right Hon Sir Zelman Cowen AK, GCMG, GCVO
Vice Chancellor Professor Richard Larkins AO

**Memo**

**To:** **Prof. Gail Risbridger, MIMR**
**From:** Glenda Johnson
**Date:** 20 March 2007
**Subject:** Notice of Approval – Project No. MMCA 2006/45

Are inhibin-$\alpha$ and VEGF-C responsible for spread of cancer cells in our model of advanced prostate cancer?

Please find attached a copy of the Final Approved proposal.

The project is approved from 01/01/07 to 31/12/2010 subject to the following conditions.

1. An Annual Report must be provided each January.
2. A Final Report is submitted to the MMC Animals Ethics Committee within six months of completion of the project.
3. Unexpected or adverse events, which impact on the welfare of the animals, must be immediately reported to the Chairperson of the AEC.
4. Any changes to location of animal housing or experimental location details are to be forwarded immediately to the AEC.
5. Special Conditions - None
6. Special Responsibilities by Animal House Staff – None
7. Approved Animal Usage is:-

| SPECIES | Total No. |
|---|---|
| Mouse SCID<br>6-8 week old/ male | 140 |

Please destroy any previous versions of the proposal and replace with the enclosed authorized document.

Regards,

Glenda Johnson
MMC Animal Ethics Committee 'A'
MIMR, Level 3
MMC Clayton
Tel. 9594-7342
Email. Glenda.johnson@med.monash.edu.au

# APPENDIX 5: SCERH APPROVAL

MONASH University

Standing Committee on Ethics in Research Involving Humans (SCERH)
Research Office

Prof Gail Risbridger
Department of Centre for Urological Research
Faculty of Medicine, Nursing and Health Sciences
Monash Medical Centre Campus

16 May 2007

**CF07/0854 - 2007/0223HT: Protein expression patterns in human prostate cancer tissues**

Dear Researchers,

Thank you for the information provided in relation to the above project. The items requiring attention have been resolved to the satisfaction of the Standing Committee on Ethics in Research Involving Humans (SCERH). Accordingly, this research project is approved to proceed.

**Terms of approval**

1. This project is approved for five years from the date of this letter and this approval is only valid whilst you hold a position at Monash University.
1. It is the responsibility of the Chief Investigator to ensure that all information that is pending (such as permission letters from organisations) is forwarded to SCERH, if not done already. Research cannot begin at any organisation until SCERH receives a letter of permission from that organisation. You will then receive a letter from SCERH confirming that we have received a letter from each organisation.
2. It is the responsibility of the Chief Investigator to ensure that all investigators are aware of the terms of approval and to ensure the project is conducted as approved by SCERH.
3. You should notify SCERH immediately of any serious or unexpected adverse effects on participants or unforeseen events affecting the ethical acceptability of the project.
4. The Explanatory Statement must be on Monash University letterhead and the Monash University complaints clause must contain your project number.
5. **Amendments to the approved project:** Changes to any aspect of the project require the submission of a Request for Amendment form to SCERH and must not begin without written approval from SCERH. Substantial variations may require a new application.
6. **Future correspondence:** Please quote the project number and project title above in any further correspondence.
7. **Annual reports:** Continued approval of this project is dependent on the submission of an Annual Report. Please provide the Committee with an Annual Report determined by the date of your letter of approval.
8. **Final report:** A Final Report should be provided at the conclusion of the project. SCERH should be notified if the project is discontinued before the expected date of completion.
9. **Monitoring:** Projects may be subject to an audit or any other form of monitoring by SCERH at any time.
10. **Retention and storage of data:** The Chief Investigator is responsible for the storage and retention of original data pertaining to a project for a minimum period of five years.

All forms can be accessed at our website www.monash.edu.au/research/ethics/human/index.html

We wish you well with your research.

Mrs Lyn Johannessen
Acting Human Ethics Officer (on behalf of SCERH)

Cc: Dr Preetika Balanathan, Prof Rob Sutherland, Dr Elizabeth Williams

Postal - Monash University, VIC 3800, Australia
Building 3E, Room 111, Clayton Campus, Wellington Road, Clayton
Telephone +61 3 9905 5490 Facsimile +61 3 9905 1420
Email scerh@adm.monash.edu.au www.monash.edu.au/research/ethics/human/index.html
CRICOS Provider No. 00008C ABN 12 377 614 012

# APPENDIX 6: TGFβ – KEYSTONE SYMPOSIA ABSTRACT

**Shift in the tumor suppressive activity of inhibin-α subunit during the transition from androgen-dependent to androgen-independent prostate cancer**

Preetika Balanathan[1], Elizabeth D Williams[2], Hong Wang[1], Marc G Achen[3], Steven A Stacker[3], Gail Risbridger[1]. [1]Centre for Urological Research, [2]Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. [3]Ludwig Institute for Cancer Research, Post Office Box 2008, Royal Melbourne Hospital, Melbourne, Victoria, Australia.

The inhibin field has been perplexed by the information that inhibin-α subunit (INHA), a member of the TGFβ superfamily is a tumor suppressor in mice yet is elevated in women with ovarian cancer. Similarly we have observed up- and down-regulation of INHA expression in prostate cancer (PCa) dependent on the stage of disease. We proposed that INHA is tumor suppressive in androgen-dependent (AD) stage of the disease but loses its tumor suppressive activity or gains metastatic properties in androgen-independent (AI) stage of the disease. Recently, loss of TGFβ receptor RIII (TGFβRIII), a receptor for inhibin has been proposed to be an explanation for the different activities of INHA in PCa.

We evaluated the functional role of INHA in two well known PCa cell lines which differ in behavior and molecular makeup and have close resemblance to primary prostate disease. The AD, LNCaP and AI PC3 cell lines were stably transfected with cDNA for INHA and evaluated for their sensitive to INHA expression in the presence of endogenous levels of TGFβRIII. Over-expression of INHA in AD LNCaP cells decreased cell proliferation and migration and reduced tumor growth supporting the role of INHA as a tumor suppressor. In contrast, over-expression of INHA in AI PC3 cells increased cell proliferation, migration, tumor growth and metastasis. This supports the loss of tumor suppressive activity or gain in metastatic properties for INHA in AI stage of the disease. The shift in the tumor suppressive activity of INHA was further evident by increase in lymph node metastasis in the INHA over-expressing PC3 tumors which was accompanied by an elevation of lymphatic vessel density and tumor cell invasion into lymphatics. These effects were associated with up-regulation of the lymphangiogenic growth factor, VEGF-C. Consistent with other studies our work revealed that LNCaP cells expressed significantly more TGFβRIII mRNA than PC3 cells.

Our results demonstrate that tumor suppressive activity of INHA is altered during the transition from AD to AI PCa. It provides the first functional evidence which suggests that loss in the tumor suppressive activity of INHA in different stages of prostate disease may be due to loss in TGFβRIII expression.

*Financial Support:* These studies were supported by Australian National Health and Medical Research Council program grant and a Post-doctoral training award by United States Department of Defense [PB, Grant#: PC060112].

# APPENDIX 7: LORNE CANCER ABSTRACT

**Tumor suppressive activity of inhibin-α subunit is altered during the transition from androgen-dependent to androgen-independent prostate cancer**

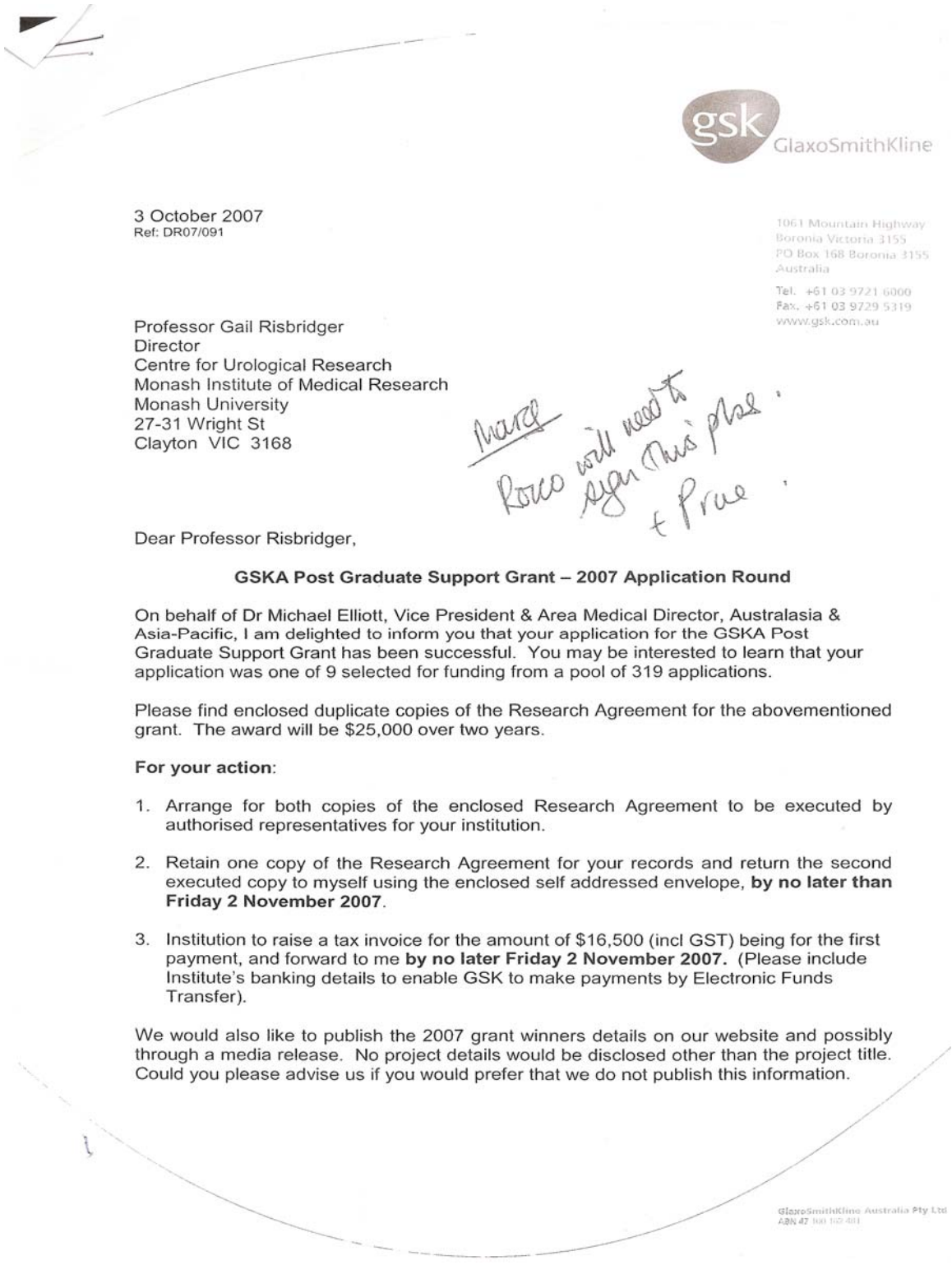
Preetika Balanathan[1], Elizabeth D Williams[2], Hong Wang[1], Marc G Achen[3], Steven A Stacker[3], Gail Risbridger[1]. [1]Centre for Urological Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. [2]Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. [3]Ludwig Institute for Cancer Research, Post Office Box 2008, Royal Melbourne Hospital, Melbourne, Victoria, Australia.

The transition from androgen-dependent (AD) to androgen-independent (AI) disease is a key event in prostate cancer (PCa) progression and Inhibin-α subunit (INHA) has been proposed to have a tumor suppressive and pro-metastatic role during different stages of the disease. Recently, loss of TGFβ receptor RIII (TGFβRIII), a receptor for inhibin has been proposed to be an explanation for the different activities of INHA in PCa.

The AD, LNCaP and AI PC3 cell lines were evaluated for their sensitive to INHA expression in the presence of endogenous levels of TGFβRIII. Over-expression of INHA in AD LNCaP cells decreased cell proliferation, migration and reduced tumor growth supporting the role of INHA as a tumor suppressor. In contrast, over-expression of INHA in AI PC3 cells increased cell proliferation, migration, tumor growth and metastasis supporting the loss of tumor suppressive activity/gain in metastatic properties for INHA in AI stage of the disease. The shift in the tumor suppressive activity of INHA was further evident by increase in lymph node metastasis in the INHA over-expressing PC3 tumors which was accompanied by an elevation of lymphatic vessel density, tumor cell invasion into lymphatics and up-regulation of VEGF-C. Consistent with other studies our work revealed that LNCaP cells expressed significantly more TGFβRIII mRNA than PC3 cells. Analysis of human PCa specimens showed that INHA expression cannot be used to determine lymph node status in PCa patients. However, increase in INHA expression by normal epithelium and prostate intraepithelial neoplasia (PIN) regions of the tumors in patients with lymph node metastasis suggests that INHA may have a paracrine role that, directly or indirectly, promotes the spread of cancer cells from the primary prostate tumor to the lymph nodes.

Our results demonstrate that tumor suppressive activity of INHA is altered during the transition from AD to AI PCa. It provides the first functional evidence which suggests that loss in the tumor suppressive activity of INHA in PCa progression may be due to loss in TGFβRIII expression.

# APPENDIX 8: GSK GRANT



GlaxoSmithKline

3 October 2007
Ref: DR07/091

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Tel. +61 03 9721 6000
Fax. +61 03 9729 5319
www.gsk.com.au

Professor Gail Risbridger
Director
Centre for Urological Research
Monash Institute of Medical Research
Monash University
27-31 Wright St
Clayton VIC 3168

Marg
Rocco will need to sign this also.
+ Prue

Dear Professor Risbridger,

**GSKA Post Graduate Support Grant – 2007 Application Round**

On behalf of Dr Michael Elliott, Vice President & Area Medical Director, Australasia & Asia-Pacific, I am delighted to inform you that your application for the GSKA Post Graduate Support Grant has been successful. You may be interested to learn that your application was one of 9 selected for funding from a pool of 319 applications.

Please find enclosed duplicate copies of the Research Agreement for the abovementioned grant. The award will be $25,000 over two years.

**For your action**:

1. Arrange for both copies of the enclosed Research Agreement to be executed by authorised representatives for your institution.

2. Retain one copy of the Research Agreement for your records and return the second executed copy to myself using the enclosed self addressed envelope, **by no later than Friday 2 November 2007**.

3. Institution to raise a tax invoice for the amount of $16,500 (incl GST) being for the first payment, and forward to me **by no later Friday 2 November 2007.** (Please include Institute's banking details to enable GSK to make payments by Electronic Funds Transfer).

We would also like to publish the 2007 grant winners details on our website and possibly through a media release. No project details would be disclosed other than the project title. Could you please advise us if you would prefer that we do not publish this information.

I would like to take this opportunity to congratulate you and Preetika Balanathan on your successful application. GSK looks forward to hearing about the progress on your exciting project.

Yours sincerely,
**GlaxoSmithKline Australia Pty Ltd**

**Ashley Bates, PhD**
**Head of R&D Alliances Aust/NZ**

Encl.

cc: Preetika Balanathan

# APPENDIX 9: ANZ GRANT

ANZ Trustees
Philanthropy Partners
Level 4, 100 Queen Street, Melbourne Vic 3000
GPO Box 389D, Melbourne Vic 3001
Telephone 03 9273 6799
Facsimile 03 9273 6354

15 October 2007

PROCESSED

Dr Preetika Balanathan
Monash University
Monash Institute of Medical Research
27-31 Wright Street
Clayton Vic 3168

Dear Dr Balanathan

| | |
|---|---|
| Project name: | **Dr Preetika Balanathan, Professor Gail Risbridger - Dual or multi-functionality of inhibin-a subunit in prostate cancer progression** |
| Organisation: | **Monash University** |
| Application date: | **29 June 2007** |
| Reference number: | **CT 9012** |

On behalf of the Trustees of **Medical Research & Technology in Victoria - The William Buckland Foundation**, I am pleased to advise that your organisation has been granted an amount of **$15,000** to be used towards the project described in your application.

With this letter we have included:

- Grant conditions
- Advice on acknowledging The William Buckland Foundation
- Reporting format

The grant is to be expended in Victoria only. Banking the enclosed cheque indicates acceptance of the grant for the purpose of the project outlined in your application and the grant conditions attached to this letter.

As set out in condition 7, your organisation is required to submit annual progress reports and a final report (or only a final report in the case of a one year project) on the project.

The Trustees wish you every success with the project and look forward to watching the progress of your project.

Yours sincerely

Trisha Broadbridge
Manager, Philanthropy Partners, ANZ Trustees Limited

Enc

40041 / 0402

# APPENDIX 10: TRAVEL SCHOLARSHIP

KEYSTONE SYMPOSIA

Connecting the Scientific Community

28 January, 2008

Preetika Balanathan
Centre for Urological Research
Monash Institute of Medical Research, Monash University
27-31 Wright Street
Melbourne, Victoria 3168 Australia

Dear Preetika Balanathan:

Congratulations on winning a scholarship for up to $1000.00 for reimbursement of costs associated with travel to the Keystone Symposia meeting TGF-ß Family in Homeostasis and Disease to be held at Eldorado Hotel & Spa, Santa Fe, New Mexico on February 3 - 8, 2008.

Immediately upon your return home, mail your original registration, air travel, ground transportation and conference lodging receipts totaling up to $1000 to me using the pre-addressed envelope. Remember, we do not reimburse meals, incidentals or recreational expenses.

I recommend that you don't use "express check-out" upon departure from the hotel. It is very difficult to get an original receipt after you have left. It would be best to pick up your itemized receipt before leaving the hotel. If you are sharing accommodations, ask for a separate receipt with your name and portion of the hotel bill.

We can accept copies of the receipts *only* if we are reimbursing your institute, in which case, please include any identifying information; i.e. name to whom the check should be made payable, department name or account number, etc. We can also accept print outs from air travel arrangements made on the internet if they include your name, departure, destination, dates and the cost. All receipts submitted should include this information. Other allowable expenses are meeting registration, ground transportation from the airport to the meeting, car rental or mileage if using a personal vehicle. We will not reimburse credit card receipts or statements so be sure to obtain all original receipts at time service is rendered. If a wire transfer is requested a $20.00 USD fee will be deducted from the award. The cost incurred by us is $40.00 USD.

Reimbursement checks will be processed as soon as *all* receipts from *all* scholarship winners have been received. **If I do not hear from you nor receive your receipts in our office on or before 21 *February 2008* your scholarship award will be forfeited. Please do not delay in sending your receipts; it will be unfair to the others.**

When sending your receipts please go into your account on our website www.keystonesymposia.org and in "Student/Postdoc Scholarship Application" under "Award Information" note the mailing address to which you would like us to mail your reimbursement check to. You can check the status of your receipts in your account as well, e.g. "Not Received", "Received", "Processed", "Payment Sent".

Please do not call our office to check on the status of the receipts. You can check it on our website n your account in "Student/Postdoc Scholarship Application" under "Receipt Status", e.g. "Not Received", "Received", "Processed", "Payment Sent". This information gets updated regularly.

Please also note that reimbursement can be issued only in your name or in the name of your institute.

If you have any questions, you can contact me at 1-800-253-0685 ext.140 or
970-262-1230 ext.140 or at ksenias@keystonesymposia.org.

Enjoy the conference!

Ksenia Shambarger
Scholarships

Program Development & Implementation • 221 Summit Place #272 • PO Box 1630 • Silverthorne, CO 80498
800-253-0685 • 970-262-1230 • 970-262-0311 (fax) • programs@keystonesymposia.org • www.keystonesymposia.org